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PEOPLE (NTC03447678), a phase II trial to test pembrolizumab as first-line treatment in patients with advanced NSCLC with PD-L1 <50%: a multiomics analysis

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ABSTRACT

Background Chemoimmunotherapy represents the standard of care for patients with advanced non-small cell lung cancer (NSCLC) and programmed death-ligand 1 (PD-L1) <50%. Although single-agent pembrolizumab has also demonstrated some activity in this setting, no reliable biomarkers yet exist for selecting patients likely to respond to single-agent immunotherapy. The main purpose of the study was to identify potential new biomarkers associated with progression-free-survival (PFS) within a multiomics analysis.

Methods PEOPLE (NTC03447678) was a prospective phase II trial evaluating first-line pembrolizumab in patients with advanced EGFR and ALK wild type treatment-naïve NSCLC with PD-L1 <50%. Circulating immune profiling was performed by determination of absolute cell counts with multiparametric flow cytometry on freshly isolated whole blood samples at baseline and at first radiological evaluation. Gene expression profiling was performed using nCounter PanCancer IO 360 Panel (NanoString) on baseline tissue. Gut bacterial taxonomic abundance was obtained by shotgun metagenomic sequencing of stool samples at baseline. Omics data were analyzed with sequential univariate Cox proportional hazards regression predicting PFS, with Benjamini-Hochberg multiple comparisons correction. Biological features significant with univariate analysis were analyzed with multivariate least absolute shrinkage and selection operator (LASSO).

Results From May 2018 to October 2020, 65 patients were enrolled. Median follow-up and PFS were 26.4 and 2.9 months, respectively. LASSO integration analysis, with an optimal lambda of 0.28, showed that peripheral blood natural killer cells/CD56dimCD16+ (HR 0.56, 0.41–0.76, p=0.006) abundance at baseline and non-classical CD14dimCD16+monocytes (HR 0.52, 0.36–0.75, p=0.004), eosinophils (CD15+CD16–) (HR 0.62, 0.44–0.89, p=0.03) and lymphocytes (HR 0.32, 0.19–0.56, p=0.001) after first radiologic evaluation correlated with favorable PFS as well as high baseline expression levels

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ In advanced non-small cell lung cancer (NSCLC), the lack of driver molecular alterations and programmed death-ligand 1 (PD-L1) Tumor Proportion Score levels (≥ or <50%) represents the standard tool to candidate patients to first-line single-agent immunotherapy or to immunotherapy and chemotherapy combinations. A subgroup of patients with advanced NSCLC and PD-L1 <50% might benefit from first-line pembrolizumab single agent, though biomarkers able to identify these patients are still lacking.

WHAT THIS STUDY ADDS

⇒ Through a multiomics approach, this study identified immune circulating cell subsets (mainly natural killer cells at baseline) and tumor tissue expression levels of genes (interferon-responsive factor 9, cartilage oligomeric matrix protein, killer cell lectin like receptor B1, protein tyrosine phosphatase receptor type C and *CD244*) positively associated to progression-free-survival in PD-L1 <50% advanced NSCLC treated with first-line pembrolizumab.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study provides a promising strategy for identification of biomarkers useful for selecting patients with PD-L1 <50% advanced NSCLC for de-escalation of chemoimmunotherapy to single-agent immunotherapy. These preliminary data need confirmation in larger studies.

of CD244 (HR 0.74, 0.62–0.87, p=0.05) protein tyrosine phosphatase receptor type C (HR 0.55, 0.38–0.81, p=0.098) and killer cell lectin like receptor B1 (HR 0.76, 0.66–0.89, p=0.05). Interferon-responsive factor 9 and cartilage oligomeric matrix protein genes correlated with unfavorable PFS (HR 3.03, 1.52–6.02, p 0.08 and HR 1.22, 1.08–1.37, p=0.06, corrected). No microbiome features were selected.

Conclusions This multiomics approach was able to identify immune cell subsets and expression levels of genes associated to PFS in patients with PD-L1 <50% NSCLC treated with first-line pembrolizumab. These preliminary data will be confirmed in the larger multicentric international I3LUNG trial (NCT05537922).

Trial registration number 2017-002841-31.

BACKGROUND

Immunotherapy (IT) is a mainstay of treatment in patients with metastatic and locally advanced non-small cell lung cancer (aNSCLC) without driver alterations.[1] Despite many limits, programmed death-ligand 1 (PD-L1) Tumor Proportion Score (TPS) still remains the only used and approved predictor of activity.[2] Tumor mutational burden (TMB) was also prospectively assessed in several randomized controlled trials[3 4]; however, its value is still debatable in effectively predicting overall survival (OS). In clinical practice, the lack of driver molecular alterations and a PD-L1 TPS ≥50% represents the standard condition to candidate patients to first-line single-agent IT or to IT-chemotherapy combinations (although it depends on regulatory rules applied among different countries).[5] In patients with PD-L1 TPS <50% IT monotherapy has shown minor activity, while IT-chemotherapy combinations based on platinum regimens proved to significantly prolong patients OS compared with chemotherapy alone, both for squamous and non-squamous histology.[6 7] However, in some countries, IT single agent is also approved in patients with aNSCLC with PD-L1 TPS 1–49%, based on KEYNOTE-042 trial results. In this study pembrolizumab improved OS in patients with aNSCLC with PD-L1 TPS ≥1% compared with standard chemotherapy.[8] Although in this trial the positive results were mostly driven by patients with PD-L1 TPS ≥50%, in the real-life clinical practice IT-chemotherapy combinations may lead to higher incidence of adverse events compared with IT single agent; therefore, pembrolizumab remains a feasible first-line choice also in patients with low PD-L1. Enhancing patient selection remains a crucial point. Unfortunately, all these treatment strategies were developed in all-comer populations (any PD-L1 expression), where either PD-L1 expression or TMB were considered only in subgroup analyses reducing the statistical significance.

Immune biomarkers are fundamentally different from oncogene biomarkers since they are continuous rather than categorical (binary) parameters, spatially variable, and reliant on multiple complex interactions rather than a single, dominant determinant. Beside PD-L1 and TMB, many biomarkers have been explored to perform more personalized diagnosis and consequently more individualized treatment decisions for patients with aNSCLC,[9 10] but results did not reach enough power to be practise changing. Different challenges remain and one of the most interesting to yet solve is surely the de-escalation of chemotherapy in patients with aNSCLC with PD-L1 TPS <50% subgroup who potentially are responsive to first-line IT single agent.

The main purpose of PEOPLE trial was to assess new biomarkers on tumor tissue, immune circulating and microbiome associated with treatment efficacy in patients with aNSCLC with PD-L1 TPS <50% treated with first-line pembrolizumab single agent. Here we report the multiomics biomarker analysis.

METHODS

Study design and patients

PEOPLE (NTC03447678) is a prospective, monocentric, open-label phase II trial conducted at Fondazione IRCCS Istituto Nazionale dei Tumori in Milan (INT). The main inclusion criteria were diagnosis of stage IIIB/IV EGFR and ALK wild type aNSCLC, who had not received prior systemic chemotherapy for advanced disease, and with PD-L1 TPS expression <50%. The availability of tissue (archival or newly obtained), blood and stool samples was mandatory for the inclusion in the trial. Full inclusion and exclusion criteria have already been described elsewhere.[11] The local Ethical Committee approved the trial protocol and all related amendments. The trial was conducted in accordance with the International Conference on Harmonization Guidelines on Good Clinical Practice and the Declaration of Helsinki. All patients provided their written informed consent before enrollment.

Treatment and procedures

Patients were enrolled from May 31, 2018, to October 07, 2020. Treatment with pembrolizumab at a flat dose of 200 mg was administered intravenously every 3 weeks (21±3 days) and continued until 2 years or 35 cycles (whichever occurred later) or until documented disease progression, unacceptable toxicity or withdrawal of the patient's consent. Tumor response was assessed every 9 weeks (63±3 days) according to the Response Evaluation Criteria in Solid Tumors (RECIST) V.1.1. PD-L1 expression was centrally assessed during the screening phase by immunohistochemistry with the anti-PD-L1 antibody (DAKO 22C3) on archival or newly obtained tumor-biopsy specimens (within 45 days prior treatment initiation) and only patients with a PD-L1 TPS expression <50% were eligible. Baseline tumor tissue was used for RNA immune gene-expression profiling. Blood samples were collected at baseline, every two cycles, and at the end of treatment for circulating immune profiling analysis. Stool samples were collected at baseline, after the first cycle and at the end of treatment.

Objectives

The primary objective of PEOPLE trial was to identify immune biomarkers associated with

progression-free-survival (PFS). Secondary objectives were: to detect differences in immune biomarkers distribution between pre-study and post-study treatment; to estimate the activity of pembrolizumab first-line monotherapy in terms of objective response rate (ORR), duration of response (DoR) and disease control rate (DCR); to assess the efficacy of the treatment in terms of OS.

Statistical analyses

The median follow-up was estimated using the inverse Kaplan-Meier (KM) method. The survival curves were estimated by the KM method.

To identify candidate biomarkers/features in the data sets obtained with each technique, a first step of sequential univariate Cox proportional hazard regression model was used for predicting PFS, taking into account the low number of patients. This procedure was applied singularly to all four set of features: circulating immune profiling at baseline(bCIP), circulating immune profiling post-immunotherapy (pCIP), gene expression profiling (GEP) and microbiome. To decrease the false discovery rate, the Benjamini-Hochberg procedure was used to correct multiple comparisons. To select the statistically significant features within each omics analysis, an alpha=0.05 was used for circulating immune profiling, while an alpha=0.1 for the microbiome and RNA expression. Correlation matrix was used to visualize the correlation between the features selected in univariate analysis and with the main relevant clinical ones. To find the optimal lambda for PFS prediction for the least absolute shrinkage and selection operator (LASSO) a 10-fold cross-validation on the previously selected features from all four modalities was performed. After this step a further feature selection using LASSO was made among the most important ones using the optimal lambda. All analyses were done with the R programming language and 'glmnet' package.

Omics methodology

Circulating immune profiling

The methodology for circulating immune profiling has been already extensively described in a previous publication.[11] Absolute cell counts of 36 immune subsets in peripheral blood were obtained using flow cytometry. Blood was collected at baseline and at day 63±3 days (first radiological evaluation) and stained with fluorescently labeled antibodies in Trucount Absolute Counting Tubes (Becton Dickinson). Samples were analyzed using a 10-color cytometer and data were analyzed with FlowJo software. Beads were gated out and peripheral blood mononuclear cells (PBMCs) were gated using side scatter (SSC) versus CD45 dot plots. Absolute cell count was calculated using the formula A=X/Y×N/V, where X=number of positive cells events, Y=number of bead events, N=number of beads in the test tube and V=test vol. Validation of flow cytometry cell counts data was obtained through correlation analysis with counts of lymphocytes, granulocytes and monocytes generated on the same samples by an automated hematology cell counter. Thirty-six distinct immune cell subsets were identified and counted using the gating strategy described in Lo Russo *et al*.[11]

GEP

Total RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor tissue at baseline, using the RNeasy FFPE Kit (Qiagen, Maryland, USA), according to manufacturer's instructions. RNA 150 ng was used for gene expression analysis performed by means of the nCounter PanCancer Immune IO 360 Panel (NanoString). The 770-plex assay panel contains: 109 genes to cell surface markers capable of quantitating 24 different tumor infiltrating immune cell types and populations, 30 genes for commonly studied antigens, over 500 genes for measuring immune response with a special emphasis on checkpoint regulation/signaling and 40 PanCancer reference genes. The *NanoStringNorm* package for R software was used to assess quality and process the data; the geometric mean of the counts relative to each sample, the mean plus two SD and the total sum of counts options were used to correct the data for technical, background and batch effect issues, respectively. The expression counts of housekeeping genes and quantile normalization were used to account for inter sample variations with the panel.

Microbiome

Metagenomic analyses were performed as described in a previous publication.[12] Briefly, DNA was extracted from frozen stool samples collected from patients before the treatment using the MOBIO PowerSoil DNA Isolation Kit (MOBIO Laboratories) and epMotion 5075 liquid handling robot (Eppendorf). DNA libraries were prepared using the Nextera DNA Flex Library Prep Kit, quantified using Qubit, and sequenced on the NovaSeq System (Illumina) using the 2×150 base pair (bp) paired-end protocol. Trimmomatic V.0.36 was used for quality trimming and adapter clipping of raw reads. The reads were then aligned against the human genome with Bowtie 2 V.2.3.2 and unaligned (non-host) reads were then assembled using MEGAHIT V.1.2.9. Assembly contigs smaller than 500 bp were discarded. Taxonomic classification of contigs was obtained by k-mer analysis using Kraken 2, with a custom 96 Gb Kraken 2 database built using draft and complete genomes of all bacteria, archaea, fungi, viruses, and protozoa available in the National Center for Biotechnology Information (NCBI) GenBank in January 2022, in addition to the human and mouse genomes. Taxonomy was expressed as the last known taxon (LKT), being the taxonomically lowest unambiguous classification determined for each sequence, using Kraken's confidence scoring threshold of 5e-06 (using the confidence parameter). Reads were aligned back to contigs, and the relative abundance for each LKT within each sample was obtained by dividing the number of bp covering all contigs pertaining to that LKT by the total number of non-host base pairs sequenced for that sample. Relative abundances were expressed in parts per million (PPM).

RESULTS

Patients' characteristics

This report is based on the final data analysis (database locked on July 22, 2021, data previously published.[11] Briefly, from May 31, 2018, to October 07, 2020, 87 treatment naïve patients with aNSCLC diagnosis, were screened in INT. Among 87 patients screened, 17 were declared screening failure and 65 were enrolled and treated. The median age was 70 years (47–87), with 44 (68%) men and 21 (32%) women. Eastern Cooperative Oncology Group Performance Status (ECOG PS) was 0 in 23 (35.4%), 1 in 30 (46.2%), and 2 in 12 (18.5%) patients. Most patients (50, 77%) had an adenocarcinoma histology while 10 (15%) patients had a squamous histology and 5 (8%) other types of histology. PD-L1 TPS expression was 1–49% in 47 (73%) patients and 0 in 18 (27%) patients. Twenty-eight (43.1%) patients received antibiotics during study treatment.

Biomarkers investigated beyond PD-L1

Three different omics evaluations have been performed, generating four different set of features. As reported in a previous publication, scanty samples or inadequate sample quality were the main reasons for not evaluating all the patients within each data set.[11]

Circulating immune profiling at baseline (bCIP): 36 immune subsets evaluated in 57 patients.

Circulating immune profiling post pembrolizumab (pCIP): 36 immune subsets evaluated in 46 patients.

Gene expression profiling (GEP) generated 778 features in 48 patients.

Microbiome: 492 gut bacterial taxa were obtained in 54 patients.

Survival analysis

At the time of analysis, with a median follow-up of 26.4 months (mo), 51 (78%) patients experienced progression and 46 (70%) died. The median PFS was 2.9 mo (95% CI 1.8 to 5.8) and the median OS was 12.1 mo (95% CI 9.0 to 20.2). Seven patients experienced an early death (before the first radiological evaluation), while 58 patients underwent at least one radiological evaluation. The ORR was 24.1%, DCR was 53.4% and the median DoR was 14.5 mo (95% CI 8.4 to 24.9).

Univariate feature analysis

Table 1 reports the features in each of the four data sets significantly correlated with PFS in the univariate analysis.

In the bCIP data sets, 3 out of 36 immune subsets were significantly and positively correlated with PFS: CD56+, natural killer (NK) cells and NK cells/CD56dimCD16+, with the following HR of: 0.5 (0.32–0.78, p=0.0293), 0.54 (0.39–0.76, p=0.006) and 0.56 (0.41–0.76, p=0.006), respectively. In the pCIP, 17 immune subsets significantly associated with PFS. Among them, the most significant with a p value ranging from 0.0008 to 0.0061 included granulo-/HLA-DRdimCD14– (HR 0.28, 0.15–0.50, p=0.0008), CD3+ (H3 0.35, 0.20–0.60, p=0.0018), lymphocytes (CD3+ or CD19+ or CD56+) (HR 0.32, 0.19–0.56, p=0.001), non-classical CD14dimCD16+ (HR 0.52, 0.36–0.75, p=0.0039), non-classical CD14dimCD16+/HLA-DR++ (HR 0.56, 0.41–0.77, p=0.0036), NK cells (HR 0.55, 0.38–0.79, p=0.0061) and NK cells/CD56dimCD16+ (HR 0.61, 0.46–0.83, p=0.0061). Of note, granulo-/HLA-DRdimCD14– is a subset including T lymphocytes (predominantly) and also NK, from which activated T cells (HLA DR+T cells) and B lymphocytes (constitutively HLA-DR+) were excluded. In the GEP data sets, expression of 21 genes significantly associated with PFS, including *CD48* (HR 0.59, 0.47–0.75, p=0.0146), *CD84* (HR 0.59, 0.45–0.77, p=0.0486), *CD244* (HR 0.74, 0.62–0.87, p value=0.0533), *CD3D* (HR 0.61, 0.47–0.80, p value=0.0533) and *killer cell lectin like receptor B1 (KLRB1)* (HR 0.76, 0.66–0.89, p value=0.0533). In the microbiome data set, the *Eisenbergiella massiliensis* species is the only taxon significantly associated with PFS (HR 13.9, 3.51–55.3, p=0.0891) in microbiome data sets.

Correlations among features

A correlation matrix was created to illustrate correlation among features significantly associated with PFS in univariate analysis (figure 1). Clinical variables such as age, smoking habits, and PD-L1 generally lack a strong correlation with features identified in the univariate analysis, although sex was slightly correlated with the expression levels of most genes. High performance status by ECOG was negatively correlated with high expression of *CD48*, *protein tyrosine phosphatase receptor type C* (*PTPRC*), *CD3D*, *CD45RA*, *CD69*, as well as elevated levels of NK and CD56+ cells, both at baseline and post-pembrolizumab. Tumor histology was correlated with the expression of a half of genes significant by univariate analysis, as well as with NK cells/CD56dimCD16+ after two cycles of therapy.

The expression levels of all the genes, except *interferon-responsive factor 9* (*IRF9*), were highly correlated with one another; most genes were positively correlated, apart from *CD276*, *proteasome 20S subunit beta 5* and *cartilage oligomeric matrix protein* (*COMP*) which were negatively correlated with the remaining genes. Additionally, the expression levels of *CD48*, *PTPRC*, *CD69*, *CD3D*, *KLRB1*, *CCL19*, *CD45RA*, *CD2*, *CD244* were positively correlated with lymphocytes subset and negatively correlated with granulocytes and neutrophils post-pembrolizumab subset.

E. massiliensis, was strongly inversely correlated with post-immune subsets, mainly with lymphocytes and NK cells.

Multiomic feature selection with LASSO

All features significant in univariate analysis were pooled for a multiomic analysis, with feature selection performed with LASSO (figures 2 and 3). Optimal λ was determined through 10-fold cross-validation, and this value (0.28) was then used for the final analysis. Among circulating biomarkers, the multiomic LASSO analysis selected NK cells/CD56dimCD16+ at baseline and granulo-/HLA-DRdimCD14–, non-classical CD14dim CD16+ and

Table 1 Multiomic features significantly associated with progression-free-survival in univariate analysis. P values are adjusted per Benjamini-Hochberg procedure

Variable	HR (95% CI)	Adjusted p value
Circulating immune profiling at baseline		
CD56+	0.5 (0.32 to 0.78)	0.0293
NK cells	0.54 (0.39 to 0.76)	0.006
NK cells/CD56dimCD16+	0.56 (0.41 to 0.76)	0.006
Circulating immune profiling post pembro		
CD3+	0.35 (0.2 to 0.6)	0.0018
CD19+	0.71 (0.54 to 0.93)	0.0301
CD19+/HLA-DR+	0.71 (0.54 to 0.93)	0.0301
CD56+	0.53 (0.33 to 0.87)	0.0301
NK cells	0.55 (0.38 to 0.79)	0.0061
NK cells/CD56brCD16dim	0.68 (0.49 to 0.94)	0.04
NK cells/CD56dimCD16+	0.61 (0.46 to 0.83)	0.0061
Intermediate CD14+CD16+/HLA-DR+++	0.62 (0.42 to 0.92)	0.0355
Non-classical CD14dimCD16+	0.52 (0.36 to 0.75)	0.0039
Non-classical CD14dimCD16+/HLA-DR++	0.56 (0.41 to 0.77)	0.0036
CD16+	2.49 (1.29 to 4.84)	0.0269
CD15hi	2.44 (1.21 to 4.9)	0.0301
Neutrophils (CD15+CD16+)	2.49 (1.28 to 4.83)	0.0269
Eosinophils (CD15+CD16–)	0.62 (0.44 to 0.89)	0.0301
Granulo-/HLA-DRdim CD14–	0.28 (0.15 to 0.5)	0.0008
Granulocytes (CD15+ or CD16+)	2.43 (1.21 to 4.9)	0.0301
Lymphocytes (CD3+ or CD19+ or CD56+)	0.32 (0.19 to 0.56)	0.001
Gene expression profiling		
CD48	0.59 (0.47 to 0.75)	0.0146
CD84	0.59 (0.45 to 0.77)	0.0486
CD244	0.74 (0.62 to 0.87)	0.0533
CD3D	0.61 (0.47 to 0.8)	0.0533
KLRB1	0.76 (0.66 to 0.89)	0.0533
CD276	2.91 (1.58 to 5.39)	0.0653
ITGA4	0.54 (0.37 to 0.78)	0.0653
COMP	1.22 (1.08 to 1.37)	0.0653
CD69	0.63 (0.48 to 0.83)	0.0653
CD2	0.57 (0.42 to 0.79)	0.0653
CCL19	0.83 (0.74 to 0.93)	0.0653
CD4	0.63 (0.48 to 0.83)	0.0653
ICOS	0.8 (0.7 to 0.92)	0.0653
SH2D1A	0.72 (0.6 to 0.87)	0.0653
IRF9	3.03 (1.52 to 6.02)	0.0833
ZAP70	0.7 (0.56 to 0.88)	0.0889
PSMB5	2.17 (1.33 to 3.54)	0.0909
JAK3	0.64 (0.48 to 0.86)	0.098
CD45RA	0 to 85 (0.77 to 0.95)	0.098
PTPRC	0.55 (0.38 to 0.81)	0.098
CD1C	0.83 (0.74 to 0.94)	0.098

Continued

Table 1 Continued

Variable	HR (95% CI)	Adjusted p value
Microbiome		
LKT_s_Eisenbergiella_massiliensis	13.93 (3.51 to 55.33)	0.0891

COMP, cartilage oligomeric matrix protein; IRF9, interferon-responsive factor 9; KLRB1, killer cell lectin like receptor B1; LKT, last known taxon; NK, natural killer; PSMB5, proteasome 20S subunit beta 5; PTPRC, protein tyrosine phosphatase receptor type C.

eosinophils (CD 15+CD16–) after two pembrolizumab cycles, all of which were associated with a favorable PFS. Five genes were selected, with higher expression levels of *CD244*, *PTPRC* and *KLRB1* genes associating with a favorable PFS and *IRF9* and *COMP* gene expression associating with poorer PFS. No microbiome features were selected by LASSO in the final integration (figure 4).

DISCUSSION

To date, clinicians have a variety of first-line treatment options for patients with aNSCLC with PD-L1 TPS <50%. Nevertheless, nowadays treatment decisions cannot accurately be taken, due to the complexity of the immune system, its interaction with the tumor microenvironment (TME) and the lack of highly accurate biomarkers. This

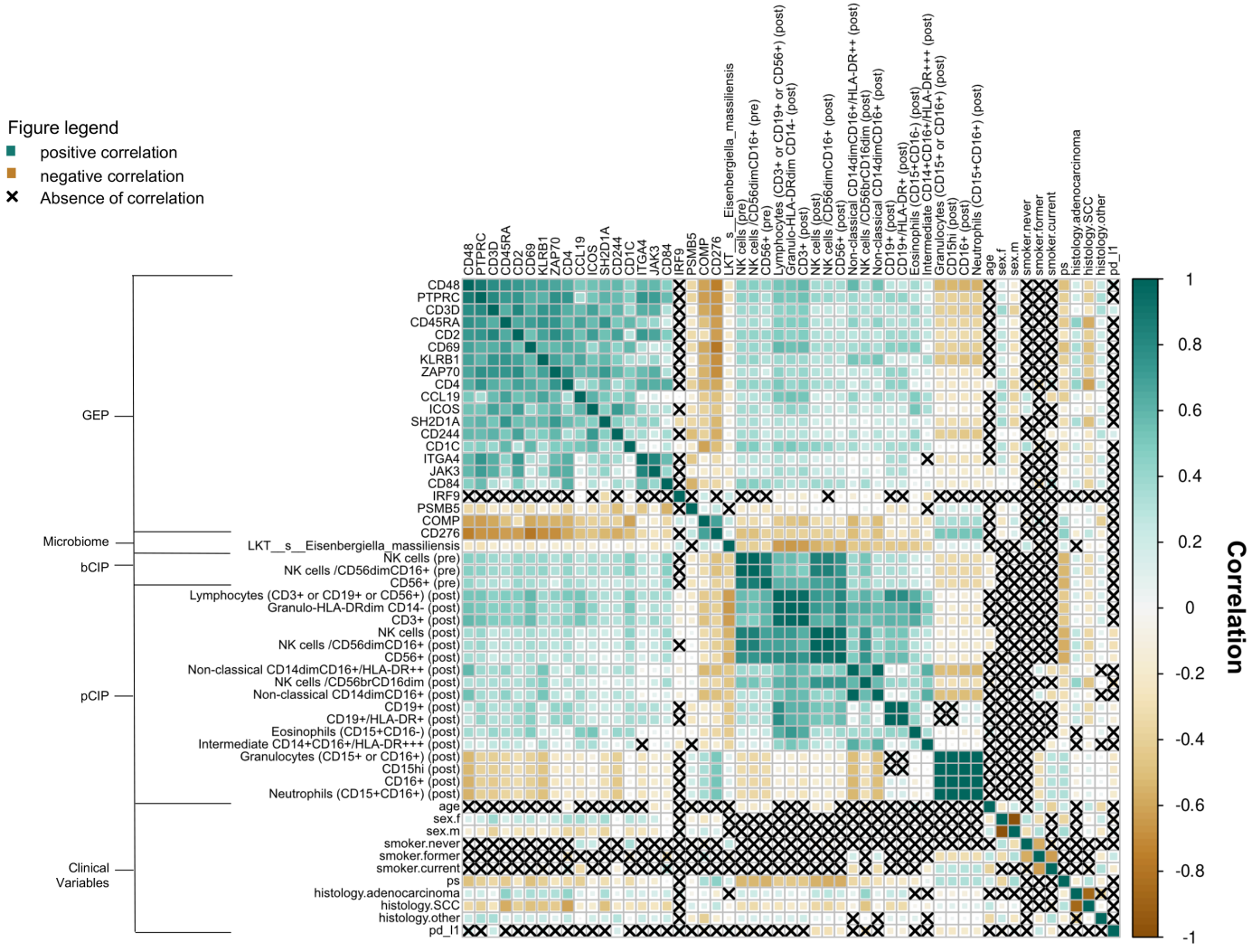

Figure 1 The correlation matrix visualizes the correlation between the features selected at univariate analysis and the most relevant clinical features. Green boxes indicate positive correlation, golden boxes indicate negative correlation, X indicates absence of correlation. bCIP, circulating immune profiling at baseline; COMP, cartilage oligomeric matrix protein; GEP, gene expression profiling; IRF9, interferon-responsive factor 9; KLRB1, killer cell lectin like receptor B1; LKT, last known taxon; NK, natural killer; pCIP, circulating immune profiling post-immunotherapy; PSMB5, proteasome 20S subunit beta 5; PTPRC, protein tyrosine phosphatase receptor type C; SCC, squamous cell carcinoma.

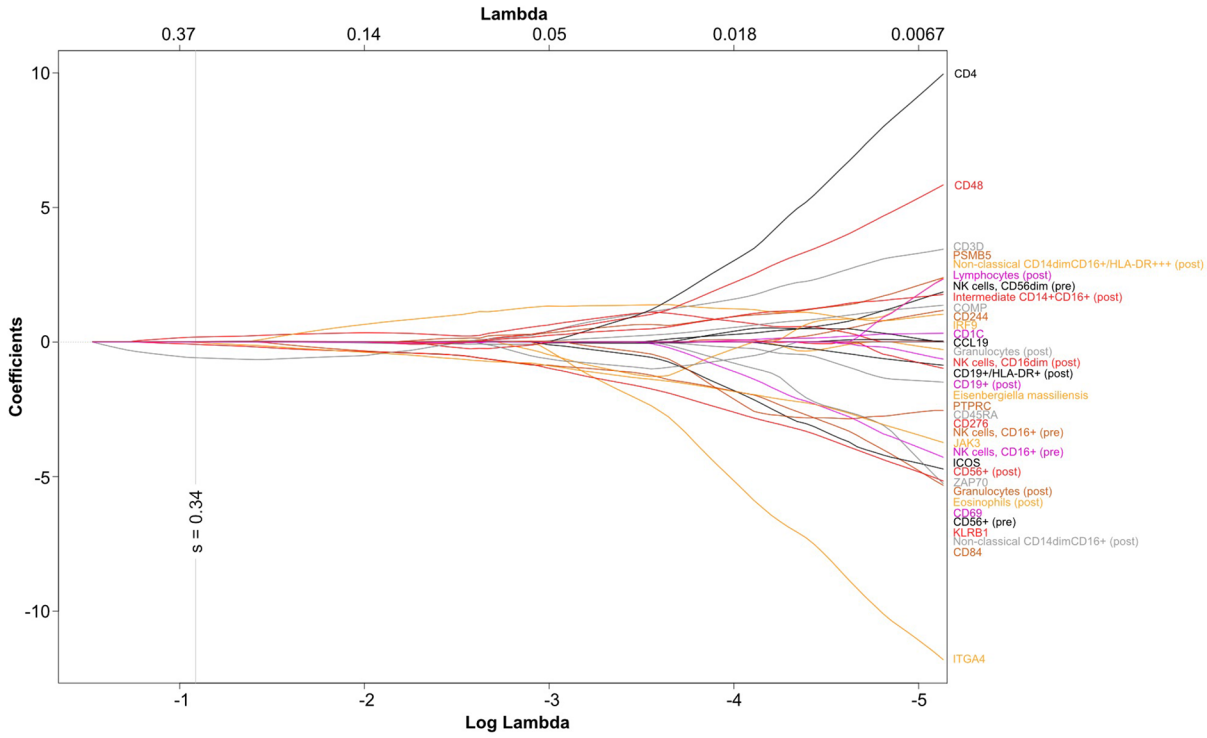

Figure 2 Feature selection using LASSO algorithm: LASSO coefficient profile for all features selected using univariant analysis. A vertical line was drawn at the optimal lambda value selected using the 10-fold cross-validation process. COMP, cartilage oligomeric matrix protein; IRF9, interferon-responsive factor 9; KLRB1, killer cell lectin like receptor B1; LASSO, least absolute shrinkage and selection operator; NK, natural killer; PSMB5, proteasome 20S subunit beta 5; PTPRC, protein tyrosine phosphatase receptor type C.

unmet need, led to the design of the PEOPLE study in 2017 and has now led to the carrying out of the present multiomic analysis.

The characterization of 36 immune subpopulations was carried out both at baseline and during pembrolizumab treatment. The final analysis selected only one subset at baseline (NK cells/CD56dimCD16+) and three subsets post pembrolizumab treatment (granulo-/HLA-DRdimCD14−, non-classical CD14dim CD16+ and eosinophils CD15+CD16−). The levels of all these subsets were positively associated with PFS. These data confirmed our previously published results, validating and strengthening our previously reported evidence on the association between high frequency of T cells (programmed cell death protein-1 (PD-1)+, CD4+) and NK cells, with improved PFS in patients with aNSCLC treated with first-line pembrolizumab single agent.[11] The potential predictive value of elevated NK cells for PFS challenges the widely held belief that immune checkpoint blockade depends primarily on T-cell activity. Indeed, several pieces of evidence from clinical and preclinical studies highlight the crucial role of innate immune cells in the context of PD-1 targeted therapy. According to Barry *et al*, NK cell frequency at the tumor site is correlated with stimulatory dendritic cells (DCs) and with higher OS and responsiveness to anti-PD-1 therapy in patients with melanoma.[13] Interestingly, Dong *et al* found that patients with PD-L1 negative tumors can still respond to IT with anti-PD-L1, but that this response is conditioned by PD-L1 positive NK cells.[14] Hsu *et al* showed in preclinical models that inhibition of PD-1 and PD-L1 promotes an NK cell response, which is necessary for IT efficacy.[15] Granulo-/HLA-DRdimCD14− is a subset of immune cells including T lymphocytes (predominantly) and also NK, from which activated T cells (HLA DR+T cells) and B lymphocytes (constitutively HLA-DR+) were excluded. Its positive correlation with PFS confirmed our previous published results highlighting the importance of T and NK cells beyond B cells.[11] Krieg *et al*, reported that CD14+CD16−HLA-DRhi monocytes were able to strongly predict PFS at baseline IT.[16] Non-classical CD14dimCD16+, differently expressed in literature as 'nonclassical patrolling monocytes', seem to play an important role in patrolling the vascular endothelial during chronic inflammatory injury with high ability to remove damaged cells[17] confirming their positive role after pembrolizumab administration. Finally, as reported in our final LASSO model, it is largely described in literature that an increased level of eosinophils cells after IT stimulation was associated with better disease control and survival outcomes.[18–20]

Looking at GEP selected genes: *COMP* and *IRF9* in our final LASSO model negatively correlate with PFS. In particular, *IRF9* encodes for a transcription factor in the type I interferon (IFN) receptor signaling pathway.[21] Interestingly, preclinical studies have demonstrated that IRF-9 dependent signaling may be involved in induction of PD-L1 expression in mouse and human lung cancer cells exposed to IFN-β[22] and of PD-1 expression in murine

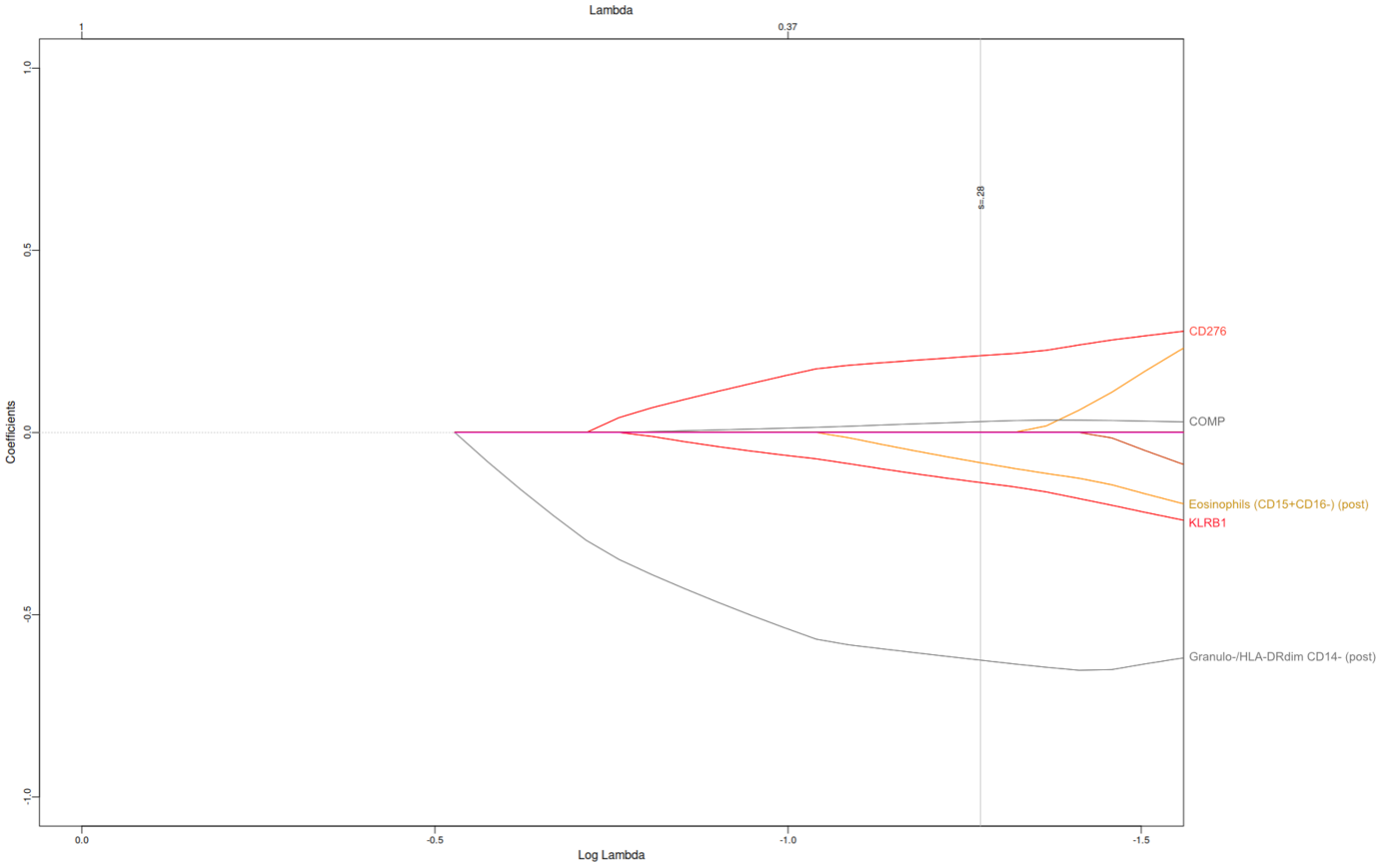

Figure 3 Feature selection using least absolute shrinkage and selection operator algorithm: Zoom-in on optimal lambda and selected features. COMP, cartilage oligomeric matrix protein; KLRB1, killer cell lectin like receptor B1.

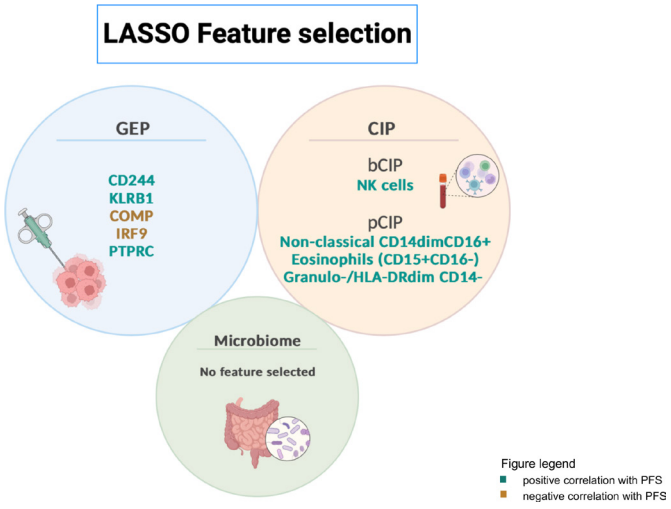

Figure 4 The figure represents all features selected by LASSO for each set of data. The green or gold color code of the features indicates a positive or negative correlation with PFS. bCIP, circulating immune profiling at baseline; CIP, circulating immune profiling; COMP, cartilage oligomeric matrix protein; GEP, gene expression profiling; IRF9, interferon-responsive factor 9; KLRB1, killer cell lectin like receptor B1; LASSO, least absolute shrinkage and selection operator; NK, natural killer; pCIP, circulating immune profiling post-immunotherapy; PFS, progression-free-survival; PTPRC, protein tyrosine phosphatase receptor type C.

T cells exposed to IFN-α.[23] Recent studies have also recognized the involvement of *IRF9* in vascular smooth muscle cells proliferation and migration through growth factors stimulation.[24 25] Therefore, we can hypothesize that the unfavorable prognostic role reported in our analysis could be driven by these two mechanisms leading to immune-escape and angiogenesis.[22–25] *COMP* encodes for a regulator of the extracellular matrix,[26] highly represented in fibrotic scars, and involved in vascular wall remodeling. Similarly, to our findings, previous studies reported a correlation with poor survival outcomes which seem to be mediated by influence on cancer cell's migration, invasion and metabolism.[27–30] On the other hand, *KLRB1*, *PTPRC* (also known as CD45) and *CD244* correlated positively with PFS in the final model. These genes encode for transmembrane receptors expressed on hematological cells. *KLRB1* is expressed on NK cells, CD8+, CD4+, and other T-cell subgroups.[31] Various studies support its favorable prognostic role in multiple tumors.[31–34] Cheng *et al* reported on 33 tumor types that *KLRB1* was positively correlated to tumor infiltrating lymphocytes, while negatively correlated to cancer-promoting myeloid cells.[31] Moreover, *KLRB1* expression reflected higher levels of immune checkpoint genes' expression, suggesting a potential capacity to predict IT response, which would be consistent with our results.[31] By the way, according to a meta-analysis performed by Braud and collaborators,

KLRB1 expression correlates with better OS in non-small cell lung cancer (NSCLC) independently from the level of tumor infiltration by CD8+T and NK cells.[34]

Elevated CD45 levels could be an index of higher tumor inflammation though they are not informative regarding the type and function of tumor infiltrate since CD45 is a pan-leukocyte protein expressed on almost all immune cells.[35] *CD244* has an immunomodulatory function and is expressed by hematopoietic cells, including NK cells, a subset of CD8+ αβ T cells, DCs and myeloid derived suppressor cells.[36–39] Few conflicting data are available regarding the impact of *CD244* expression on NSCLC outcomes. A negative prognostic role has been hypothesized by Vaes and coauthors, who reported a negative correlation of *CD244* expression levels with PFS among 26 patients with stage I NSCLC treated with stereotactic body radiation therapy.[40] However, based on this study results, high *CD244* expression levels seem to confer a positive role on PFS. This hypothesis is also supported by an exploratory analysis conducted on 14 patients with NSCLC treated with atezolizumab within POPLAR (study comparing second line atezolizumab vs docetaxel).[41]

The positive correlation between most of the immune-related genes assessed by NanoString and the peripheral immune profile suggests that a better clinical outcome to pembrolizumab depends on both peripheral and tumor-related factors. Although not surprizing, this also means that patients with the best clinical outcome need to have at the same time a systemic immune profile geared towards a functional T/NK compartment and, likely, also an inflamed/leukocyte-infiltrated TME (PTPRC, CD244, KLRB1) that is well poised to react/be functionally rescued by pembrolizumab. In other words, clinical response to pembrolizumab is a systemic process and only the integrated analysis on tumor tissue and periphery can provide such evidence.

Finally, among microbiome features, no one was selected in the final LASSO model. However, it is now well accepted that the gut microbiota is causally implicated in the efficacy and toxicity of IT in several solid tumors including aNSCLC.[42 43] In fact, fecal microbiota transplantation or dietary interventions have been clinically investigated to improve the success rate of IT.[44] However, little concordance exists among species identified in different studies. In the present study only *E. massiliensis* was found associated with PFS. Whether it depends on negative or low PD-L1 expression or on the high microbiota heterogeneity which cannot be mitigated due to the small number of patients, remains to be determined. In addition, the use of antibiotics in a subgroup of patients, reportedly associated with dysbiosis and progression on IT treatment[43] could have contributed to limit the power of the analysis. *E. massiliensis* is a gram-negative bacterium isolated from human stool that belongs to the Firmicutes phylum and Lachnospiraceae family.[45 46] Even if not selected as significant variable in multivariate analysis, its amount in the gut at baseline was highly negatively correlated with post-treatment immune features that remained in the final model—mainly lymphocytes, reportedly determinant of IT efficacy[47]—supporting its inverse association with IT activity and its putative role as modulator of the immune system. This correlation may be clinically relevant, because this bacterium was characterized in stool collected at baseline, so it anticipates the immune features that would be evaluated later. The impact of this bacterium in immune modulation and IT efficacy has not been investigated yet, but it was recently found to be increased in the gut of mice by ketogenic diet and to be highly correlated with the main-ketogenic metabolite, three hydroxybutyric acid, in the blood of mice and humans.[48] Although this metabolite, similar to butyrate, was found associated with antitumor immune modulation and IT efficacy in preclinical models,[48 49] it was also described to have anti-inflammatory and tolerogenic roles mainly in inflammatory disease,[50] supporting a context and dose dependent effects. Comprehensive metagenomic analysis and preclinical studies would shed light on the role of gut microbiota and of this bacterium in the response to anti-PD-1 therapy.

The present study has some limitations, mainly the non-randomized design and the small sample size.

Conversely, the strength of the study lies in its innovative multiomics approach, which allows the concomitant evaluation of multiple biologic sets to select the most relevant biomarkers.

The strength of the present study mainly relates to its prospective phase II biomarker-driven design including innovative multiomics approach. Compared with other first-line IT single-agent studies, the present study population was frailer with a higher proportion of patients with ECOG PS 2. Also, the study included patients with negative PD-L1 expression levels, a population that has not been included in the previous trials with single-agent IT.

Over the last years, omics technologies have experienced a sharp acceleration in their development and their costs have also been reduced.[51] But there is still one thing missing: their integration based on the consideration of the individual as a whole. Since the human mind is unable to integrate them and—far less—to correlate them with response to IT, our main ambition is to create and prospectively validate an artificial intelligence (AI) tool that allows scientists to predict more precisely the efficacy of treatment while allowing us to integrate scientific knowledge. In this perspective, the INT-led I3LUNG Horizon Europe project (https://cordis.europa.eu/project/id/101057695) aims to expand this cohort with other multiomics data collected across Europe and beyond, with the objective to achieve peak performance in personalized medicine through an AI/machine learning method modeled on multimodal patients' data.

In conclusion, to our knowledge this is the first prospective drug-interventional trial investigating the association of multiomics biomarkers and efficacy of IT within PD-L1 TPS <50% aNSCLC.

The results suggested that mainly NK cells subset evaluated at baseline IT may be useful in identifying those

patients who may benefit from pembrolizumab, avoiding the additional toxicity expected to be induced by chemotherapy. Other relevant immune subsets, evaluated in the post pembrolizumab setting, included lymphocytes, monocytes and eosinophils subsets. In addition, the evaluation of some selected genes on tumor tissue such as of *IRF9, COMP, KLRB1, PTPRC* (CD45) and *CD244* could be implemented within clinical practice to individualize selection among single agent and IT-chemotherapy combination in this subset of patients. Comprehensive metagenomic studies are required to clarify the influence of *E. massiliensis* and other metagenomic biomarkers on IT outcomes.

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